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Development of Liquid Chromatographic Method for Simultaneous Determination of Mycophenolate Mofetil and its Degradation Product Mycophenolic Acid in Dosage Form

A. Protić*, Lj. Živanović, M. Zečević, and **B. Jocić**

Institute of Pharmaceutical Chemistry and Drug Analysis, Faculty of Pharmacy, Vojvode Stepe 450, P.O. Box 146, 11 000 Belgrade, Serbia

Abstract

In this paper, a chemometrical approach is applied for the development of a reversed-phase high-performance liquid chromatography method for the simultaneous determination of mycophenolate mofetil and its degradation product mycophenolic acid in dosage form. The fractional factorial design is used in screening experiments for selecting the variables that significantly influence the chromatographic procedure. The investigated variables are column type, temperature of the column, and composition of the mobile phase (with respect to pH and the percentage of organic modifier). Investigation is performed using two columns, XTerra (RP 18, 150 mm × 3.9 mm) and Chromolith (RP-18e, 100 mm × 4.6 mm). Because the column type shows no influence on separation process, the Chromolith column is further used due to its ability to achieve a high-speed separation without loss of column efficiency. Total analysis time is reduced from 8.34 min on XTerra to 1.27 min on Chromolith. The columns' efficiency, analysis cost, and peak symmetries are briefly compared. For both substances, only two variables are found significant: percentage of acetonitrile and pH of the water phase. Afterward, the main variables are optimized using response surface methodology for visualization and easier identification of optimal conditions. The optimal conditions are obtained with mobile phase composition of acetonitrile–15mM phosphate buffer (pH adjusted to 4.0 with 85% orthophosphoric acid) (35:65, v/v) at the flow rate of 5 mL/min. The temperature of the column is adjusted to 25°C and detection is performed at 254 nm.

Introduction

Mycophenolate mofetil (MMF, Figure 1A) is an immunosuppressive drug which is most frequently used in combination with prednisone after solid organ transplantation. Also, it is often used for the treatment of renal disease due to systemic lupus erythematosus and may be useful in vasculitis and Wegener granulomatosis. On the other hand, it is occasionally administrated to treat rheumatoid arthritis, as there are few controlled data regarding its efficacy in this area.

MMF is a pro drug which is rapidly absorbed and hydrolyzed to mycophenolic acid (MPA, Figure 1B), the active metabolite (1). According to the manufacturer's specification, the hydrolysis process can also occur in dosage form during the storage of Cellcept capsules and as the level of MPA increases. Degradation products are considered as impurities which might lead to problems associated with toxicity, bioavailability, or different pharmaceutical products' performance. As the level of degradation of MMF directly influences its bioavailability, MPA is always investigated as an impurity in dosage form. At the same time, according to International Conference on Harmonization (ICH) guidelines on impurities in new drug products, identification and quantitation is necessary for all impurities above 0.1% level (2,3). The control specification for MPA in capsule formulation is up to a maximum 1%.

Considering this, a new isocratic, reversed-phase high-performance liquid chromatography (RP-HPLC) method was developed and validated for the simultaneous determination of MMF and its degradation product in dosage form. This is the first study in which quantitative analysis was performed for the simultaneous determination of MMF and MPA. In the literature is found only methods for the determination of

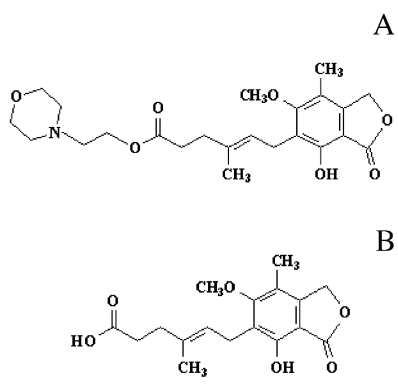

Figure 1. Structures of MMF (A) and MPA (B).

* Author to whom correspondence should be addressed: email anna@pharmacy.bg.ac.yu.

MMF or MPA in biological materials (4–11). Most of them use ion pair reagents (5,8,10,11), and require a change of flow rate (6) or even gradient elution (7). The liquid chromatography–mass spectrometry (LC–MS) method for the quantitation of MPA has also been established (12). As few laboratories are in a position to perform LC–MS, a new sensitive, precise, and rapid RP-HPLC–UV method for the determination of MMF and MPA in dosage forms in routine analysis is welcome in this area. There are no reported papers from dosage form, either.

Many studies show the performance of monolith columns (13–18) and compare these columns with conventional reversed-phase columns (19–23), but there are no papers about the performance of monolithic columns against the XTerra column. In order to decide whether to use the Chromolith or XTerra column, a brief comparison between these two columns was also made.

Experimental

Instrumentation and materials

HPLC analysis was performed with an Agilent Technologies (Palo Alto, CA) HP 1100 chromatograph equipped with HP 1100 binary pump, HP 1100 UV-visible detector, and Rheodine 20-µL loop injector. Compounds were separated on an XTerra RP 18, 150 mm × 3.9 mm, 5 µm particle size column (Waters, Milford, MA), as well as on a Chromolith RP-18e, 100 mm × 4.6 mm, macropore size 2 µm, mesopore size 13 nm (Merck, Darmstadt, Germany) column.

Water for chromatography was obtained from a Millipore (Billerica, MA) System Simplicity 185 purification system. Before use, the mobile phase and the solutions to be injected were degassed and vacuum filtered through 0.45 µm nylon membranes (Alltech Associates, Lokeren, Belgium).

Data was acquired with ChemStation software from HP. For linear regression and statistical analysis, Microsoft Excel version software was used. The polynomial equations and response surface plots were achieved from Statistica 5 software.

Drugs and materials

MMF and MPA standards were purchased from Sigma (Taufkirchen, Germany). Commercially available Cellcept capsules (one capsule contains 250 mg of MMF) were produced by Roche (Nutley, NJ).

HPLC-grade acetonitrile was obtained from Lab Scan (Dublin, Ireland), potassium dihydrogen phosphate from Merck, 85% orthophosphoric acid from Carlo Erba (Milan, Italy), and phenacetin was a U.S. Pharmacopeia reference substance.

Chromatographic conditions

The chosen mobile phase for the separation of MMF and its degradation product MPA on the XTerra column consisted of acetonitrile–15mM KH_2PO_4 buffer, in which pH was adjusted to 4.0 with 85% orthophosphoric acid (35:65, v/v). For the Monolith column, it consisted of acetonitrile–15mM KH_2PO_4 buffer, in which pH was adjusted to 4.2 with 85% orthophosphoric acid (35:65, v/v). Flow rate was 1 mL/min for XTerra and 5 mL/min for Chromolith, and detection was performed at 254 nm on both columns.

Standard and test solutions

A stock solution of MMF was prepared at a concentration of 1 mg/mL in 50:50 (v/v) acetonitrile–water as a solvent. A stock solution of MPA was prepared in the same solvent as MMF at a concentration of 0.1 mg/mL. A 0.1 mg/mL stock solution of phenacetin as internal standard was also prepared in 50:50 (v/v) acetonitrile–water.

For construction of calibration curve, standard solutions were prepared at concentrations of 0.05; 0.1; 0.2; 0.3; 0.4; 0.5 mg/mL for MMF and at concentrations of 0.5; 1; 2; 3; 4; 5 µg/mL for MPA.

Test solutions were prepared from Cellcept capsules. Capsule powder containing 25 mg of MMF was transferred to a 25-mL volumetric flask and dissolved in approximately 20 mL of 50:50 (v/v) acetonitrile–water by use of an ultrasonic bath for 15 min. The solution was then diluted to volume with the same solvent and filtered. The concentration of MMF was 1 mg/mL. This solution was used to attain a test solution in a final concentration of 0.2 mg/mL for MMF.

Standard solutions of MMF and MPA used for validation process as well as test solutions were prepared in mobile phase, with the addition of internal standard, which was added to obtain the final concentration of 10 µg/mL.

Experimental design and methodology

In the first step of the investigation, experimental design was used to determine variables which have statistically important influence on the chromatographic behavior of the investigated compounds. However, if the number of variables to be optimized becomes large, this will increase the number of experiments (24). When applying experimental design methodologies, it is advisable to keep the number of variables as low as possible in order to avoid very complex response models and large variability (25). Considering this, in the case of detailed modeling it is often desirable at a first stage to reduce the number of factors via screening design to a smaller number of main factors that are to be studied in detail (employing optimization designs), for which both squared and interaction terms in the model are of interest (26). Applying fractional factorial design, the number of experiments can be kept low, based on the assumption that interaction effects between three or more variables are small compared to main effects and two-variable interaction effects. Thus, it is possible to select a fraction of the full factorial design and omit several combinations of variables from the experimental plan (27). The number of experiments in fractional factorial design is given as $2^{k-p} + C$, where k is the number of variables, C the number of replicates, and p a whole number that indicates how fractionated the experimental design will be. When p is zero, the experimental design is full (24). The repetition of experiments provided a precise estimate of an experimental error.

The statistical model for analyzing the screening design with four factors has the following form:

$$Yx_1x_2x_3x_4 = \beta_0 + \beta_1 x_{1i} + \beta_2 x_{2i} + \beta_3 x_{3i} + \beta_4 x_{4i} + r_{1i} \quad \text{Eq. 1}$$

where Y is the response variable, β_0 is the general mean, β_1, β_2, β_3 and β_4 are the estimated factor effects, r_{1i} is the residual error, and x_1, x_2, x_3, and x_4 are the levels of each factor. In the screening design, only two levels were used so that the factors were considered as discrete variables and no continuous second order response model could be estimated. The effect of each factor was tested using a Student test with a corresponding p-value. The factors whose p-values were less than 0.05 were considered as "statistically significant". A graphical display of the ordered standardized effect (the absolute value of the estimated effect divided by its standard error estimate) of each factor was given in a Pareto chart. A factor was considered as "statistically significant" if its standardized effect exceeded a threshold. A line in the Pareto chart indicated the threshold for a test at level 0.05 (28,29).

In the second step of the optimization, response surface methodology (RSM) was applied. RSM presents a collection of mathematical and statistical techniques for analyzing the effect of several independent variables on dependent variables and provides its graphical representation. The effect of two variables can be represented as a surface in three-dimensional space and the influence of two variables on the response can be clearly seen in the investigated region. Furthermore, the response surface methodology enables the prediction of the behavior of the response between and slightly outside the investigated area, as well as visualization and rapid selection of optimal conditions (30).

When investigating the influence of two variables, the response surface might be described by some mathematical function, f, that relates the response, Y, to the levels of factors x_1 and x_2:

$$y = f(x1, x2) \quad \text{Eq. 2 (28).}$$

Results and Discussion

During development of the HPLC method, variables that could have influence on the chromatographic performance must be taken into consideration. Generally, HPLC separation depends on the physical and chemical properties of the compounds, composition and pH of the mobile phase, column temperature, and stationary phase properties. According to this, in the screening phase all variables that could influence the separation of MPA and MMF as well as their domains were determined.

During preliminary experiments, the nature of the stationary phase was firstly investigated. The C18 packing columns were shown to be the most suitable according to the nature of the compounds. The paramount of modern pharmaceutical analysis is to provide higher column efficiency and shorter analysis time. On one hand, the XTerra C18 column enabled better peak shape of the compounds than conventional C18 columns, which is because of its hybrid technology. On the other hand, monolithic packing as a new development can achieve a high-speed separation. Considering this, the previously mentioned columns were included in the experimental design to compare their influence on chromatographic separation, and afterwards the comparison of the efficiency and analysis speed was performed.

Afterwards, the percentage of organic modifier was examined. Between organic modifiers, acetonitrile showed the best characteristics considering peak shape and retention parameters. As the retention time of compounds was unreasonably prolonged when the amount of acetonitrile was below 25%, this percentage was chosen as the low level. On the other hand, 35% of organic modifier was chosen for the high level because above this percentage, no separation could be achieved.

Considering peak broadening and symmetries, the addition of phosphate buffer was necessary. Good peak symmetries were achieved with 15mM KH_2PO_4, and because the concentration of phosphate buffer showed no influence on the retention parameters, buffer concentration was not taken into consideration during further investigation.

The pH values of the water phase were varied from 2.4 to 4.8. This decision was based on the stability of MMF and good separation from its degradation product, MPA.

The temperature was examined in the range from 25 to 35°C. Because the peak symmetries of both compounds were considerably worse at higher temperatures, a wider range of temperature was not investigated.

After preliminary experiments, 2^{4-1} fractional factorial design was performed. The investigated variables and their domains are presented in Table I. High and low levels of each variable are based on the preliminary investigations and are denoted as +1 and –1. The experimental plan for fractional factorial design is reported in Table II, and it must be noted that all experiments were performed randomly and in duplicate in order to estimate the experimental error. The observed response was retention factor for both substances, and according to obtained values standardized effects were calculated. Based on experimental error and absolute values of standardized effects, the significance of examined factors was evaluated in the same way as explained in the "Experimental design and methodology" section. Pareto charts, of which the length of the bars is proportional to the absolute value of the standardized effects, are presented in Figure 2. As has already been said, a line in the Pareto chart indicated the threshold for a test at level $p = 0.05$ (for $t_{crit} = 4.303$).

The standardized effects are also presented in Figure 2 in order to see whether the influence of the variable on the response is positive or negative. If a standardized effect is a negative value, it means that an increase in a variable leads to a

Table I. Investigated Variables and their Domains

Variables	Investigated levels: Low level (–1)	High level (+1)
Column	Chromolith	XTerra
pH value of the water phase	2.4	4.8
Column temperature (°C)	25	35
Acetonitrile (%) in the mobile phase	25	35

decrease in a retention parameter and if it is positive the increase of variable leads also to increase of the response. From the results obtained, the following conclusions could be made.

The percentage of acetonitrile and pH of the water phase showed statistically important influences on the chromatographic behavior of both substances. Both variables had a negative effect on the retention factor of MPA, though the percentage of acetonitrile had a negative and pH of the water phase a positive effect on the retention parameter of MMF. These variables have been further investigated and optimized using RSM.

As could be expected, due to the fact that both columns are C18, no significant influence of the columns on the retention behavior of the investigated compounds was noticed. During experiments, it was observed that the Chromolith column provided much shorter analysis time and the XTerra column enabled better peak symmetries. In order to decide which column to use in further optimization, the columns were compared and results are presented in Table III. All parameters were obtained with the same composition of the mobile phase and were further compared. Due to the structure of the Chromolith column, analysis could be performed at a flow rate of 5 mL/min. The Chromolith column is made by sol gel technology, which enables the formation of highly porous material containing macropores and mesopores in its structure. The large pores (typically 2 μm) are responsible for a low flow resistance and therefore allow the application of high eluent flow rates, while the small pores (approximately 12 nm) ensure sufficient surface area for separation efficiency. Due to this, higher flow rates can be used while the resolution of silica rod column is much less affected in comparison to particulate materials after increasing the flow rate and maintaining low column pressure (19). So, the flow rate could be increased with no significant increase of back-pressure. On the other hand, the flow rate on the Xterra column was constant and kept at 1 mL/min. These flow rates allowed much shorter analysis times of the compounds on the Chromolith column. But this was not all: the Chromolith column also showed a higher efficiency, and a slightly smaller amount of acetonitrile was used for analysis. Based on these results, we decided to use the Chromolith column in further optimization and validation of the method.

Another variable that showed no influence on the chromatographic behavior of MPA and MMF was column temperature. The temperature was held at 25°C in further optimization. This decision was based on the previously mentioned fact that symmetries of the peaks were much better at a lower temperature.

Table II. Plan of Experiments in Fractional Factorial Design for MMF and MPA

Exp. no.	Variables A	B	C	D
1	–1	–1	–1	–1
2	+1	–1	–1	+1
3	+1	+1	–1	–1
4	+1	–1	+1	–1
5	–1	–1	+1	+1
6	+1	+1	+1	+1
7	–1	+1	–1	+1
8	–1	+1	+1	–1

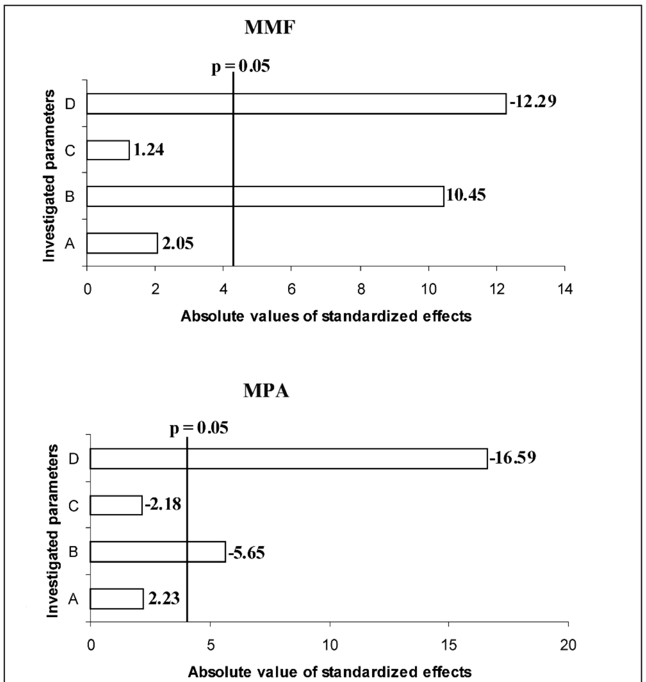

Figure 2. Pareto charts of standardized effects (absolute values) obtained from the fractional factorial design for MPA and MMF.

Table III. Performances of Chromolith Column and XTerra Column

Parameter	Monolith column	XTerra
Mobile phase	35% ACN–15Mm KH_2PO_4, pH adjusted to 4.0	35% ACN–15Mm KH_2PO_4, pH adjusted to 4.0
Flow rate (mL/min)	5	1
Analysis time (min)	1.27	8.34
Need of ACN during one run (mL)	2.22	2.92
Retention factor (k)		
MMF	1.50	1.77
MPA	3.26	6.87
N considering MMF*	8053.41	4473.64
HETP†	0.012	0.034
Peak symmetry		
MMF	1.30	1.002
MPA	0.84	0.924

* N—plate number.
† HETP—height equivalents of a theoretical plate.

Because no factor interactions and optimal conditions could be obtained from fractional factorial design, RSM was further applied.

Two variables that needed further optimization were percentage of acetonitrile in the mobile phase and pH value of the water phase. As mentioned, RSM was applied and the influences of the variables on the retention parameters are clearly shown in Figure 3. The relationship between variables and response is the best described with the following equations:

$z = 53.369 - 3.008x - 0.983y + 0.052x^2 - 0.169xy + 1.07y^2$
for MMF, and

$z = 148.977 - 8.207x - 0.712y + 0.109x^2 + 0.16xy - 0.659y^2$
for MPA,

where y is the concentration of acetonitrile, x is the pH value of the water phase, and z is the retention factor.

Not only separation, but also reasonable retention time of MPA was the goal of this optimization. Based on the response surface plots, it can be noticed that interactions exist between variables x_1 and x_2 for MMF. The higher the pH values of the water phase, the bigger the influence of the percentage of acetonitrile. This interaction is not drastically emphasized for MPA. For both substances, the percentage of acetonitrile has the biggest influence on retention time. It can be concluded that optimal retention factors for both compounds were achieved with 30% of acetonitrile. Below this percentage, the retention of the compounds are unnecessary long, especially for MPA, and also for MMF when pH of the water phase is higher than 4.0. On the other hand, when pH of the water phase is between 2.4 and 3.6, the retention factor of MPA was unreasonably high. Because of this compound, it was decided to work on higher pH values but not higher than 4.3, considering that above this pH, no baseline separation could be achieved. One more thing was noticed. Peak symmetries for both compounds were the best when pH of the water phase was 4.0. Based on all observations, the most suitable mobile phase was chosen and the mobile phase composition is described in detail in the "Chromatographic conditions" section.

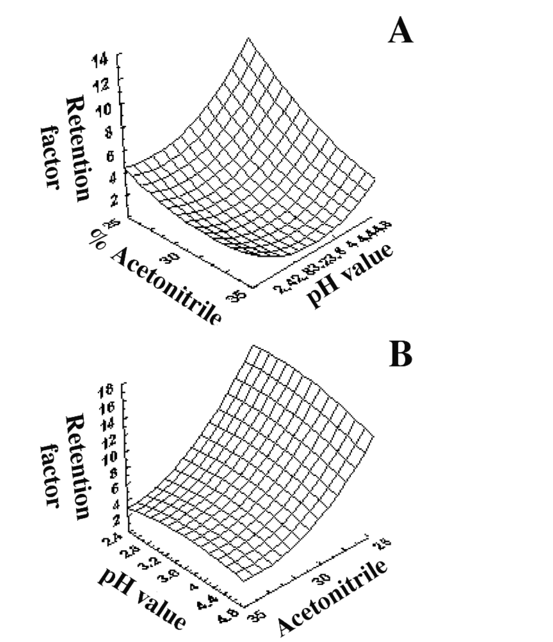

Figure 3. Three-dimensional retention factor diagrams $k_1 = f$ (% AcN, pH) for MMF (A) and MPA (B).

Validation of the optimized method

After the optimization procedure, validation was carried out according to ICH guidelines (31). During the validation process, the method was tested for selectivity, linearity, precision, limit of detection (LOD), and limit of quantitation (LOQ).

The selectivity of the method was investigated by observing potential interferences between MMF and its degradation product with tablet excipient, and no interfering peaks were noticed, which can be observed in Figure 4.

The internal standard method was used for quantitation. After the injection of every prepared solution into the HPLC system, the peak areas were measured and the ratios of the peak area of the investigated substances to that of the internal standard were calculated. The linearity of the relationships between peak areas and concentrations for MMF and MPA were investigated by analyzing standard solutions. Standard solutions were prepared in the concentration range 0.05–0.5 mg/mL for MMF and 0.5–5 µg/mL for MPA. Six solutions of MMF and MPA were prepared in the mobile phase, with addition of internal standard to yield a final concentration of 10 µg/mL, and each were injected in triplicate. Regression lines for MMF and MPA were constructed by the method of least-squares. Data from regression analysis are presented in Table IV and the difference of intercepts from zero (p = 0.05, t_{tab} =

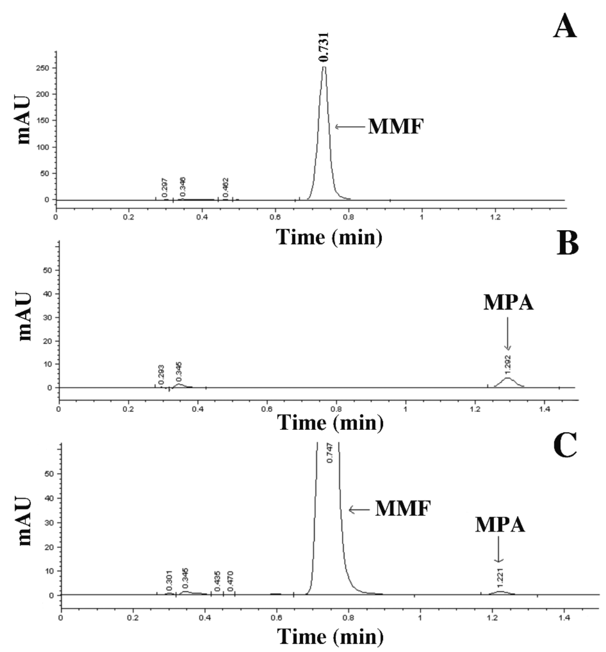

Figure 4. Representative chromatograms of working solution of MMF (A), MPA (B), and MMF and MPA from dosage form, Cellcept capsules (C) on Chromolith column. Mobile phase: ACN–15mM KH_2PO_4 buffer with pH adjusted to 4.0 with 85% orthophosphoric acid (35:65, v/v); column temperature: 25°C; flow rate: 5 mL/min; detection on 250 nm.

Table IV. Regression Analysis Data

Compound	y = ax + b	r*	Sa†	Sb†	ta‡
MMF	$26.910x - 0.340$	0.9994	2.100	0.550	2.37
MPA	$0.0374x + 0.0015$	0.9999	0.0017	0.004	1.58

* r—correlation coefficient.
† Sa, Sb—standard deviations of the slope and intercept.
‡ t—calculated deviation value for intercept.
t0.05 – 2.571

Table V. Precision of the Assay for Three Standard Solutions of Three Different Concentrations

Compound	Concentration (mg/mL)	Amount found	SD (mg/mL)	RSD (%)*
MMF	0.1	0.097	0.001	0.77
	0.2	0.198	0.001	0.68
	0.3	0.300	0.003	1.11
MPA	1	1.004	0.012	1.24
	2	1.983	0.018	0.89
	3	2.975	0.051	1.70

* RSD—relative standard deviation.

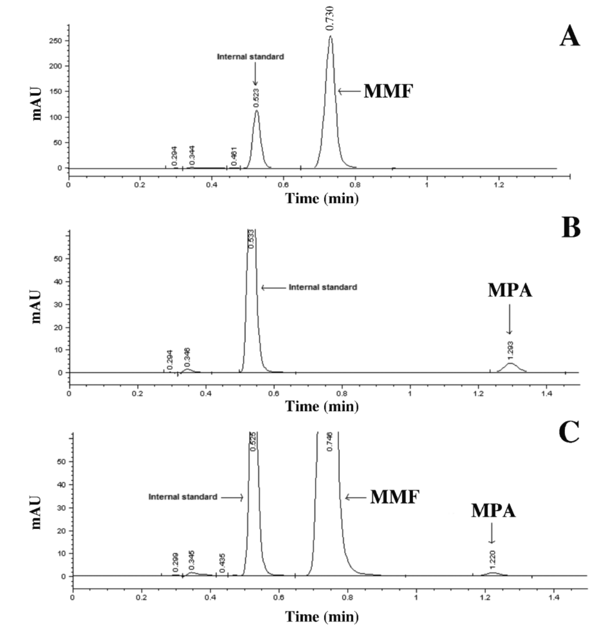

Figure 5. Representative chromatograms of working solution of MMF and phenacetin as internal standard (A), MPA and phenacetin (B), and MMF and MPA from dosage form, Cellcept capsules, and phenacetin (C) on Chromolith column. Mobile phase: ACN–15mM KH_2PO_4 buffer with pH adjusted to 4.0 with 85% orthophosphoric acid (35:65, v/v); column temperature: 25°C; flow rate: 5 mL/min; detection on 250 nm.

Table VI. Assay of MFM and MPA in Cellcept Capsules

Compound	Taken	Found	SD	RSD (%)	Found in mg/capsule	R (%)
MMF (mg/mL)	0.2	0.203	0.004	1.92	253.70	101.48
MPA (µg/mL)	2 MAC*	0.064	0.002	3.54	0.064	

* MAC—maximum allowed content.

2,571) was found to be insignificant.

The precision of the chromatographic method was investigated for three concentrations from calibration curves of each investigated analyte. Three solutions for each concentration level of analyzed compounds were freshly prepared prior to analysis. The results and important statistical data are given in Table V, and it could be concluded that the method showed satisfactory precision.

Furthermore, LOD was measured as the lowest amount of the analyte that may be detected to produce a response which is significantly different from that of a blank. The values of LOD were experimentally determined. The LOD was 0.03 µg/mL for MMF and 0.025 µg/mL for MPA.

LOQ was estimated as the lowest amount of the analyte that can be reproducibly quantitated above the baseline noise with a RSD ≤ 3% for replicated injections. Obtained values were 0.10 µg/mL for MMF and 0.09 µg/mL for MPA.

All data met the criteria from the ICH regulations. Furthermore, the applicability of the proposed method for the assay of dosage form was examined by analyzing commercially available Cellcept capsules. Chromatograms of the standard and test solutions, together with phenacetin as the internal standard, are shown in Figure 5. Obtained data from dosage form are presented in Table VI, and it could be concluded that the low RSD value indicates satisfactory repeatability of the method.

According to these results, it must be concluded that a precise, sensitive, rapid, and solvent- and time-saving method was developed on the Chromolith column.

Conclusion

With the assistance of experimental design, a new method has been developed and validated for the simultaneous determination of MMF and its degradation product MPA in dosage form. It is ideal for routine analysis considering its simplicity and short analysis time of only 1.27 min per run. Aside from this method's outstanding ability to save analysis time and solvent, during the validation process, the method also showed outstanding sensitivity, precision, and selectivity.

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